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Kinetic mechanism of vanillyl-alcohol oxidase with short-chain 4-alkylphenols

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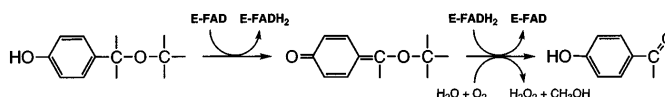
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The kinetic mechanism of vanillyl-alcohol oxidase with 4-methylphenol, 4-ethylphenol, 4-propylphenol and their $C\alpha$ -deuterated analogs has been studied at pH 7.5 and 25 °C. Conversion of 4-methylphenol is extremely slow (0.005 s^{-1}) while the enzyme is largely in the reduced form during turnover. 4-Ethylphenol and 4-propylphenol are readily converted while the enzyme is mainly in the oxidized form during turnover. The deuterium kinetic isotope effect for overall catalysis ranges between 7–10 whereas the intrinsic deuterium kinetic isotope effect for flavin reduction ranges over 9–10. With all three 4-alkylphenols, flavin reduction appeared to be a reversible process with the rate of reduction being in the same range as the rate for the reverse reaction. During the reductive half-reaction of vanillyl-alcohol oxidase with 4-ethylphenol and 4-propylphenol, a transient intermediate is formed with an absorbance maximum at 330 nm. This intermediate has been tentatively identified as the *p*-quinone methide of the aromatic substrate in complex with reduced enzyme. It is concluded that vanillyl-alcohol oxidase catalysis with 4-ethylphenol and 4-propylphenol favors an ordered sequential binding mechanism in which the rate of flavin reduction determines the turnover rate while the reduced enzyme–*p*-quinone methide binary complex rapidly reacts with dioxygen. During the reaction of vanillyl-alcohol oxidase with 4-methylphenol, a fluorescent enzyme species is stabilized. Based on its spectral characteristics and crystallographic data [Mattevi, A., Fraaije, M. W., Mozzarelli, A., Olivi, L., Coda, A. & van Berkel, W. J. H. (1997) *Structure* 5, 907–920], it is proposed that this species represents a covalent 5-(4'-hydroxybenzyl)-FAD adduct. With 4-ethylphenol and 4-propylphenol, similar N5 flavin adducts may be formed but their rate of formation is too slow to be of catalytic relevance.

Keywords: alkylphenol; flavoprotein; *p*-quinone methide; kinetic isotope effect; vanillyl-alcohol oxidase.

Vanillyl-alcohol oxidase is a covalent flavoprotein isolated from *Penicillium simplicissimum*, a filamentous fungus capable of growing on a wide variety of aromatic compounds [1, 2]. The enzyme is a rather stable homooctamer with each 65-kDa subunit containing an 8 α -(N^3 -histidyl)-FAD molecule [3, 4]. Vanillyl-alcohol oxidase is active with a wide range of *para*-substituted phenolic compounds [5], but the physiological function of the enzyme is not fully understood. Based on induction experiments, 4-(methoxymethyl)phenol has been proposed to represent the physiological substrate [2]. A detailed kinetic study with this phenolic methylether has pointed to a ternary complex mechanism in which flavin reduction is the rate-limiting step in catalysis [6]. The reaction of vanillyl-alcohol oxidase with 4-(methoxymethyl)phenol involves the initial formation of a binary complex of reduced enzyme and the *p*-quinone methide of 4-(methoxymethyl)phenol. This complex then reacts with molecular oxygen, reoxidizing the flavin, and after water addition of the *p*-quinone methide, the products 4-hydroxybenzaldehyde and methanol are formed (Eqn 1).



The reaction mechanism of vanillyl-alcohol oxidase has properties in common with that of *p*-cresol methylhydroxylase [7]. This bacterial flavocytochrome converts a wide range of 4-alkylphenols first into 4-hydroxybenzyl alcohols and subsequently into 4-hydroxybenzaldehydes. In contrast to vanillyl-alcohol oxidase, flavin reoxidation in *p*-cresol methylhydroxylase involves the transfer of electrons to a tightly bound cytochrome subunit [8].

The crystal structure of vanillyl-alcohol oxidase has recently been solved at 2.5 Å resolution [9]. Each vanillyl-alcohol oxidase monomer consists of two domains: one creates a binding site for the ADP moiety of the FAD molecule, while the other domain covers the active center which is located between the two domains. The structure shows that the isoalloxazine ring of the flavin makes a covalent bond with His422. Furthermore, the active site is located in the interior of the protein and contains an anion-binding pocket facilitating the deprotonation of phenolic substrates. Based on the structures of several vanillyl-alcohol oxidase–inhibitor complexes, it could be determined that the distance between the $C\alpha$ atom of aromatic substrates and the reactive N5 of the isoalloxazine ring is about 3.5 Å. Equally close to the $C\alpha$ atom of the inhibitors is the side chain of Asp170 the function of which is not yet clear. The structure of the vanillyl-alcohol oxidase monomer is very similar to that of the

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Enzymes. Catalase, hydrogen-peroxide oxidoreductase (EC 1.11.1.6); *p*-cresol methylhydroxylase (EC 1.17.99.1); vanillyl-alcohol oxidase (EC 1.1.3.7).

flavoprotein subunit of *p*-cresol methylhydroxylase [10, 11]. Most active-site residues are conserved but, intriguingly, Asp170 of vanillyl-alcohol oxidase is replaced by Ser in *p*-cresol methylhydroxylase [12].

The parent substrate of *p*-cresol methylhydroxylase, 4-methylphenol, is a very poor substrate for vanillyl-alcohol oxidase [13]. The crystallographic analysis of vanillyl-alcohol oxidase suggested that this might be related to the formation of a covalent adduct between the C α atom of 4-methylphenol and flavin N5 [9]. In view of this, it was of interest to address the kinetic mechanism of vanillyl-alcohol oxidase with short-chain 4-alkylphenols. In this study, 4-methylphenol, 4-ethylphenol and 4-propylphenol were used as model substrates and the deuterium kinetic isotope effects for the overall and reductive half-reactions were determined. For this purpose, an improved method for the synthesis of C α -deuterated 4-alkylphenols was developed.

MATERIALS AND METHODS

General. 4-Methylphenol, 4-ethylphenol, 4-propylphenol, methyl-4-hydroxybenzoate, 4-hydroxyacetophenone, 4-hydroxypropiophenone, LiAlD₄, *t*-butyldimethylsilyl chloride, imidazole, D₂O and AlCl₃ were from Aldrich. Glucose oxidase (grade II) was from Boehringer. Vanillyl-alcohol oxidase from *P. simplicissimum* (ATCC 90172) was purified as described before [3, 5]. Enzyme concentrations were calculated from the molar absorption coefficient of the oxidized flavin ($\epsilon_{439} = 12.5 \text{ mM}^{-1} \text{ cm}^{-1}$) [3].

Analytical methods. HPLC experiments were performed with a Lichrospher RP8 (4.6×150 mm) reverse-phase column, connected to an Applied Biosystems 400 pump and a Waters 996 photodiode-array detector. Products were eluted with methanol/water/acetic acid (33:66:1) at 1 ml min⁻¹. GC/MS analysis was performed on Hewlett Packard HP 5973 MSD and HP 6090 GC equipped with a HP-5 column. The initial temperature was 80°C. After injection, the temperature was raised at 7°C min⁻¹ up to 240°C. ¹H-NMR spectra were recorded on a Bruker AC-200 (200-MHz) spectrometer. Samples were dissolved either in (²H₅)pyridine or CDCl₃ containing tetramethylsilane as internal standard.

Steady-state kinetic experiments were carried out essentially as described earlier [5]. Kinetic experiments were performed at 25°C in 50 mM potassium phosphate, pH 7.5, unless stated otherwise. Oxygen consumption was measured with a Clark electrode [3]. Conversion of 4-ethylphenol and 4-propylphenol was followed by the increase of absorbance at 260 nm. Formation of 4-hydroxybenzaldehyde from 4-methylphenol was measured at 340 nm ($\epsilon_{340} = 10.0 \text{ mM}^{-1} \text{ cm}^{-1}$). Absorption spectra were recorded at 25°C on an Aminco DW-2000 spectrophotometer. Fluorescence emission spectra were recorded at 25°C on an Aminco SPF-500C spectrofluorometer. The excitation wavelength was 360 nm, using a bandwidth of 4 nm. For anaerobic experiments, samples were flushed with argon.

Stopped-flow kinetic studies were carried out with a Hi-Tech SF-51 apparatus equipped with a Hi-Tech M300 monochromator diode-array detector. Spectra were scanned in the 300–550-nm wavelength range with a scan time of 10 ms. Deconvolution analysis of spectral data was performed using the Specfit Global Analysis program version 2.10 (Spectrum Software Ass.). Single-wavelength kinetic traces were recorded using a Hi-Tech SU-40 spectrophotometer. All concentrations mentioned concerning stopped-flow experiments are those after mixing. In anaerobic experiments, solutions were flushed with argon and contained glucose (10 mM) and glucose oxidase (0.1 μM) to

ensure anaerobic conditions. For enzyme-monitored turnover experiments [14], air-saturated enzyme (5 μM) and substrate (1.0 mM) solutions were mixed in the stopped-flow instrument after which the redox state of the flavin cofactor was recorded at 439 nm.

Synthesis of 4-[α -²H₃]methylphenol. Methyl 4-hydroxybenzoate (4.6 g, 0.030 mol), imidazole (4.0 g, 0.060 mol) and *t*-butyldimethylsilyl chloride (5.3 g, 0.035 mol) in 120 ml dimethylformamide was stirred under nitrogen for 72 h. The mixture was subsequently diluted with 200 ml water and extracted twice with 200 ml petroleum ether (bp 40–60°C). The ether layer was washed with water and dried over magnesium sulphate. The ether was removed by evaporation, yielding a yellow oil of methyl 4-([1-*t*-butylsilyl]oxy)-benzoate, yield 96%; ¹H-NMR (CDCl₃) δ_{H} ppm = 0.3 (t, 6H, CH₃), 1.0 (s, 12H, *t*-butyl), 1.6 (s, 3H, CH₃), 6.8 (d, 2H, Ar), 7.0 (d, 2H, Ar). Mass spectrum: m/z (relative abundance) = 266 (M⁺, 10), 235 (6), 209 (100), 135 (29), 91 (15), 73(8), 59(13).

Methyl 4-([1-*t*-butylsilyl]oxy)-benzoate (4.0 g, 0.015 mol) and AlCl₃ (1.6 g, 0.012 mol) in 20 ml ether was added dropwise to a stirred solution containing 4.8 g (0.036 mol) AlCl₃ and 2.0 g (0.048 mol) LiAlD₄ in 180 ml ether. The mixture was refluxed for 70 h after which some D₂O was added to remove remaining LiAlD₄. After acidification with H₂SO₄, the solution was extracted twice with 150 ml ether. The ether was removed by evaporation and, in the final step, the deuterated product was purified by silica column chromatography (eluent: dichloromethane/ethyl acetate, 4:1) yielding 1.37 g pure 4-[α -²H₃]methylphenol (pale yellow oil): yield, 82%, ¹H-NMR (CDCl₃) δ_{H} ppm = 5.1 (s, 1H, OH), 6.7 (d, 2H, Ar), 7.0 (d, 2H, Ar). Mass spectrum: m/z (relative abundance) = 111 (M⁺, 100), 110 (68), 109 (58), 93 (7), 82 (21), 77(20), 63(4), 53(10).

Synthesis of 4-[α -²H₂]ethylphenol and 4-[α -²H₂]propylphenol. 4-4-[α -²H₂]Ethylphenol and 4-[α -²H₂]propylphenol were synthesized essentially as described above, starting from 4-hydroxyacetophenone and 4-hydroxypropiophenone, respectively. Pure 4-[α -²H₂]ethylphenol (1.62 g, yellowish crystals) was obtained with a yield of 87%; ¹H-NMR (CDCl₃) δ_{H} ppm = 1.2 (s, 3H, -CH₃), 5.1 (s, 1H, -OH), 6.8 (d, 2H, Ar), 7.1 (d, 2H, Ar). Mass spectrum, m/z (relative abundance) = 124 (M⁺, 41), 110 (12), 109 (100), 79 (13), 66 (3), 53(4). Pure 4-[α -²H₂]propylphenol (0.79 g, pale yellow liquid) was obtained with a yield of 38%, ¹H NMR (CDCl₃) δ_{H} ppm = 1.0 (t, 3H, -CH₃), 1.6 (q, 2H, -CH₂-), 5.0 (s, 1H, -OH), 6.8 (d, 2H, Ar), 7.1 (d, 2H, Ar). Mass spectrum: m/z (relative abundance) = 138 (M⁺, 34), 109 (100), 93 (3), 79 (17), 53 (4).

RESULTS

Steady-state kinetics. In a previous study we have shown that vanillyl-alcohol oxidase oxidizes a wide range of phenolic compounds with turnover rates ranging over 1–10 s⁻¹ [5]. Results in Table 1 show that both 4-ethylphenol and 4-propylphenol are converted at a similar rate. With these 4-alkylphenols and using stopped-flow spectrophotometry, it was found that during steady state the enzyme is mainly in the oxidized form. Enzyme-monitored turnover experiments revealed that, with ethylphenol, 69% of the enzyme was in the oxidized state while, with 4-propylphenol, 63% was in the oxidized state during turnover. When the deuterated isomers were used, an increase in the amount of oxidized enzyme during catalysis was observed (90% and 82% oxidized vanillyl-alcohol oxidase for 4-[α -²H₂]ethylphenol and 4-[α -²H₂]propylphenol, respectively). Furthermore, the turnover rates for these compounds revealed a relatively large deuterium kinetic isotope effect (Table 1). This suggests that, for both

Table 1. Steady-state kinetic parameters for the reaction of vanillyl-alcohol oxidase with short-chain 4-alkylphenols and 4- $[\alpha\text{-}^2\text{H}]$ alkylphenols. All experiments were performed at pH 7.5, 25 °C. Values of kinetic parameters have a standard error < 10%; n.d., not determined. Values in parentheses are the isotope effect ($^1\text{H}/^2\text{H}$).

Substrate	k_{cat}	K_{m}	$k_{\text{cat}}/K_{\text{m}}$
	s^{-1}	μM	$\text{mM}^{-1} \text{s}^{-1}$
4-Methylphenol	0.0050	31	0.16
$[\alpha\text{-}^2\text{H}_3]$ Methylphenol	0.0007 (7)	n.d. (–)	– (–)
Ethylphenol	2.5	9.0	300
$[\alpha\text{-}^2\text{H}_2]$ Ethylphenol	0.24 (10)	11 (0.8)	22 (13)
Propylphenol	4.2	3.7	1100
$[\alpha\text{-}^2\text{H}_2]$ Propylphenol	0.56 (7.5)	3.0 (1.2)	190 (6.2)

4-ethylphenol and 4-propylphenol, a step involving flavin reduction is limiting the rate of overall catalysis.

Vanillyl-alcohol oxidase converts both 4-ethylphenol and 4-propylphenol to a mixture of the corresponding benzylic alcohol and alkenylic phenol in a ratio of approximately 4:1 [15]. HPLC product analysis revealed that deuteration of C α has no effect on the ratio of aromatic products. When the reaction with 4-ethylphenol was performed in D₂O, no significant effect was seen on the ratio of aromatic products as well. However, using equimolar amounts of H₂O and D₂O, a 15% increase of the rate of turnover was observed.

Conversion of 4-methylphenol by vanillyl-alcohol oxidase leads to formation of 4-hydroxybenzaldehyde as final aromatic product [13]. Analogously to *p*-cresol methylhydroxylase catalysis [16], this reaction involves two consecutive substrate-oxidation steps. However, as can be seen from Table 1, vanillyl-alcohol oxidase converts 4-methylphenol at an exceptionally low rate. Therefore, the enzyme was mixed with 4-methylphenol under aerobic conditions, and the redox state of the flavin was monitored spectrophotometrically. It was found that the enzyme is mainly (58%) in the reduced state during turnover (Fig. 1, spectrum 2) while product is continuously formed as evidenced by the increase in absorbance at 330 nm (Fig. 1, spectrum 3). Moreover, in separate experiments, oxygen consumption could be detected over a long time, confirming continuous turnover. The apparent reduction of the flavin during turnover was even more pronounced at higher pH values and, at pH 9.4, vanillyl-alcohol oxidase became almost fully reduced (Fig. 1, spectrum 4). The absorption spectrum of the 4-methylphenol-reduced enzyme species was strikingly different from free reduced enzyme [6] and showed a typical absorbance maximum at 352 nm with a molar absorption coefficient of 7.6 mM⁻¹ cm⁻¹. Recently, evidence was obtained from X-ray crystallography that vanillyl-alcohol oxidase can form a stable 4-methylphenol–flavin N5 adduct [9]. Therefore, the apparent reduction of the flavin may reflect formation of this adduct and its slow decay might limit the turnover rate (see also below). As the enzyme is mainly in a reduced state during catalysis at pH 7.5, the relatively large isotope effect on k_{cat} (Table 1) is difficult to interpret. Apparently, a reaction step leading to oxidized enzyme is rate limiting and its velocity is influenced by deuterium replacement of the C α hydrogens of 4-methylphenol. When vanillyl-alcohol oxidase was mixed with deuterated 4-methylphenol, an increase of oxidized enzyme was observed (77% in the oxidized state), sug-

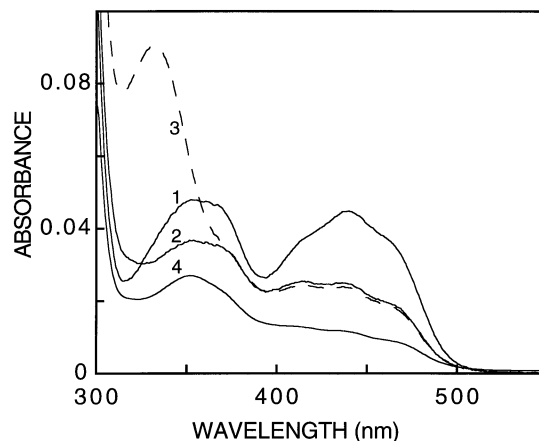
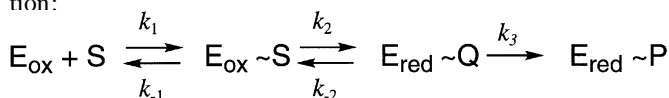


Fig. 1. Spectral changes observed upon aerobic reaction of vanillyl-alcohol oxidase with 4-methylphenol. Vanillyl-alcohol oxidase (3.6 μM) was aerobically mixed with 4-methylphenol (500 μM) and spectra were recorded after reaching an apparent equilibrium of the redox state. Spectra: (1) before the addition of 4-methylphenol; (2) after a 1-min incubation at pH 7.5; (3) after a 7.5-min incubation at pH 7.5; (4) after a 1-min incubation at pH 9.4, 50 mM glycine/NaOH. Spectra were recorded at 25 °C.

gesting that the rate of enzyme reduction is partially limiting the rate of overall catalysis.

Reductive half-reaction. The reductive half-reaction of vanillyl-alcohol oxidase can be described by the following equation:



where E_{ox} represents oxidized enzyme, E_{red} represents reduced enzyme, S represents substrate, Q represents intermediate product and P represents the final product.

When vanillyl-alcohol oxidase and 4-ethylphenol were mixed in the stopped-flow spectrophotometer under anaerobic conditions, a fast decrease of absorbance at 439 nm indicative for flavin reduction was observed. The reaction traces could satisfactorily be fitted assuming a biphasic process with rate constants at saturating substrate concentrations $k_{\text{fast}} = 3.6 \text{ s}^{-1}$ and $k_{\text{slow}} = 0.3 \text{ s}^{-1}$, respectively. Furthermore, the slow component (k_{slow}) was not dependent on the substrate concentration while the other component (k_{fast}) reached a finite value at the lowest substrate concentrations measured (Fig. 2). The secondary process was too slow to account for the turnover rate (2.5 s⁻¹) and therefore is probably of no catalytic importance. When the kinetic data for k_{fast} were analyzed, a best fit was obtained using the procedure of Strickland et al. [17] which includes an apparent initial reduction rate at infinite low substrate concentrations indicating that reduction is a reversible process ($k_{-2} > 0$, Eqn 2). By this approach a reduction rate of 2.5 s⁻¹ (k_2) was determined while the reversible step occurs at a rate of 1.1 s⁻¹ (k_{-2}) (Fig. 2, Table 2). The subsequent slow process reflects the decay of the formed binary complex of E_{red}~Q (k_3 in Eqn 2).

With 4- $[\alpha\text{-}^2\text{H}_2]$ ethylphenol, again a biphasic reduction was observed with markedly decreased rates for both steps ($k_{\text{fast}} = 1.3 \text{ s}^{-1}$ and $k_{\text{slow}} = 0.05 \text{ s}^{-1}$ at a substrate concentration of 0.5 mM). However, accurate determination of the rate of the fast phase was hampered by the fact that the absorbance decrease, and therefore the extent of reduction in the first phase, was sig-

Table 2. Kinetic parameters for the reductive half-reaction of vanillyl-alcohol oxidase with short-chain 4-alkylphenols. All experiments were performed at pH 7.5, 25°C. Rates for deuterated substrates were determined from the ratio of $[E]_{ox}/[E]_{total} [= k_{-2}/(k_{-2} + k_2)]$, with $k_{fast} = k_2 + k_{-2}$ at the end of the first reductive process. Values in parenthesis are the isotope effect ($^1H/^2H$).

Substrate	k_2 s^{-1}	k_{-2}	$K_d (= k_{-1}/k_1)$ μM	k_3 s^{-1}
Methylphenol	0.076 ± 0.015	0.086 ± 0.013	42 ± 15	—
$[\alpha\text{-}^2H_3]$ Methylphenol	0.008 ± 0.002 (9.5)	0.04 ± 0.01 (2)	n.d. (—)	— (—)
Ethylphenol	2.5 ± 0.3	1.1 ± 0.2	10 ± 2	0.3
$[\alpha\text{-}^2H_2]$ Ethylphenol	0.3 ± 0.1 (10)	1.0 ± 0.1 (1.1)	n.d. (—)	0.05 (6)
Propylphenol	4.4 ± 0.4	1.6 ± 0.3	0.8 ± 0.2	1
$[\alpha\text{-}^2H_2]$ Propylphenol	0.5 ± 0.1 (9)	1.6 ± 0.2 (1.0)	n.d. (—)	0.2 (5)

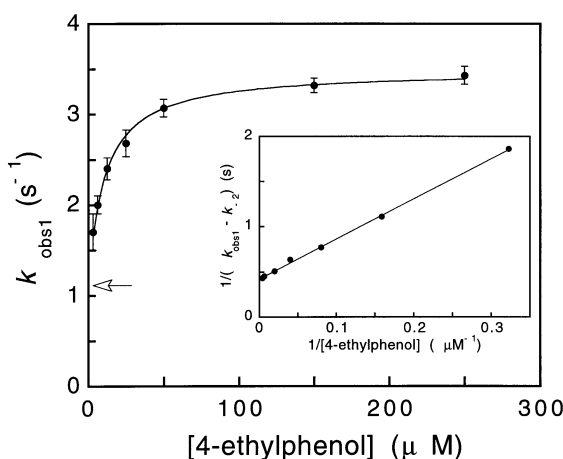


Fig. 2. Observed reduction rates (k_{fast}) of vanillyl-alcohol oxidase with varying concentrations of 4-ethylphenol. The anaerobic reduction experiments were performed at 25°C and pH 7.5. Flavin reduction was monitored at 439 nm. Each determined reduction rate ($k_{obs} = k_{fast}$) is an average of 15 analyzed traces. The arrow indicates the value found for the reverse reduction rate. In the inset the double-reciprocal plot of the kinetic data (corrected for k_{-2}) is shown.

nificantly decreased. Furthermore, both rate constants appeared to be independent of the substrate concentration. This again is in line with a model in which the first step represents a reversible reduction of the flavin. With 4- $[\alpha\text{-}^2H_2]$ ethylphenol, the slow rate of reduction results in an equilibrium in which most of the enzyme is in the oxidized state as $k_2 < k_{-2}$ and hence a small decrease in absorbance during the first phase is observed. Using the fraction of oxidized enzyme present at the end of the first reductive step [corresponding to $k_2/(k_{-2} + k_2)$], both k_2 and k_{-2} can be determined. For this, we determined the apparent reduction rate ($k_{fast} = k_2 + k_{-2}$) and the redox state of the enzyme at the end of the reduction, at saturating conditions. Using this method, we could calculate the reduction rate ($k_2 = 0.3 s^{-1}$) and the rate of the reverse step ($k_{-2} = 1.0 s^{-1}$), the latter being similar to the rate found with 4-ethylphenol (Table 2). Except for the intrinsic isotope effect on the reduction rate k_2 , a relatively large isotope effect on k_3 was also found. A similar sequence of reactions and corresponding isotope effects have been observed during the reductive half-reaction of D-amino acid oxidase from yeast [18]. In that case, it was proposed that the second process

represents a step involving substrate conversion or product dissociation or results from coincidence of kinetic rates.

To verify the reversibility of reduction of vanillyl-alcohol oxidase by 4-ethylphenol and to monitor spectral changes during the reductive half-reaction, diode-array detection was used. Fig. 3A shows the spectral course of the anaerobic reduction of vanillyl-alcohol oxidase by 4-ethylphenol. Analysis of these data clearly revealed that an initial fast reductive process is followed by a relatively slow secondary process (Fig. 3B). Except for the decrease of absorbance at 439 nm, indicative for flavin reduction, also a marked increase in absorbance around 330 nm was observed during the first process (spectrum 2; Fig. 3B). Based on studies with 4-(methoxymethyl)phenol [6], this intermediate spectrum ($\epsilon_{330} = 25 mM^{-1} cm^{-1}$) is ascribed to the formation of a binary complex between reduced enzyme and the *p*-quinone methide of 4-ethylphenol. This is substantiated by data of Bolton et al. [19] who have synthesized a *p*-quinone methide of a 4-ethylphenol analogue which exhibits an absorbance maximum at 322 nm. Using the fraction of oxidized enzyme formed after the first process (spectrum 2 in Fig. 3B), the rate of reduction (k_2) and of the reverse reaction (k_{-2}) could be determined (assuming that spectrum 3 in Fig. 3B represents a fully reduced enzyme species). Using the absorbance values at 439 nm (Fig. 3A), k_2 and k_{-2} were $2.6 s^{-1}$ and $0.8 s^{-1}$, respectively. These values agree well with the data obtained using the single-wavelength acquisition mode. In the second slow process the absorbance at 330 nm decreased (spectrum 3, Fig. 3B) and a spectrum was formed which shows some resemblance with the 4-methylphenol-generated reduced enzyme species (spectrum 4, Fig. 1).

Similar results as observed for the reductive half-reaction with 4-ethylphenol were obtained when vanillyl-alcohol oxidase was anaerobically mixed with 4-propylphenol. Again, the kinetic data were consistent with a model which includes reversible reduction ($k_{-2} > 0$). For 4-propylphenol, a relatively low value for the dissociation constant of the Michaelis complex (k_{-1}/k_1) was found while all other kinetic parameters, including the isotope effects on k_2 and k_3 , were in the same range as found for 4-ethylphenol (Table 2). The transient intermediate formed with 4-propylphenol had an absorbance maximum around 330 nm and from the kinetic analysis a molar absorption coefficient, $\epsilon_{330} = 22 mM^{-1} cm^{-1}$ could be calculated. This, and the fact that the *p*-quinone methide of a 4-propylphenol analogue exhibits an absorbance maximum at 326 nm [20], supports the idea that this intermediate represents the initial formation of the *p*-quinone methide of 4-propylphenol.

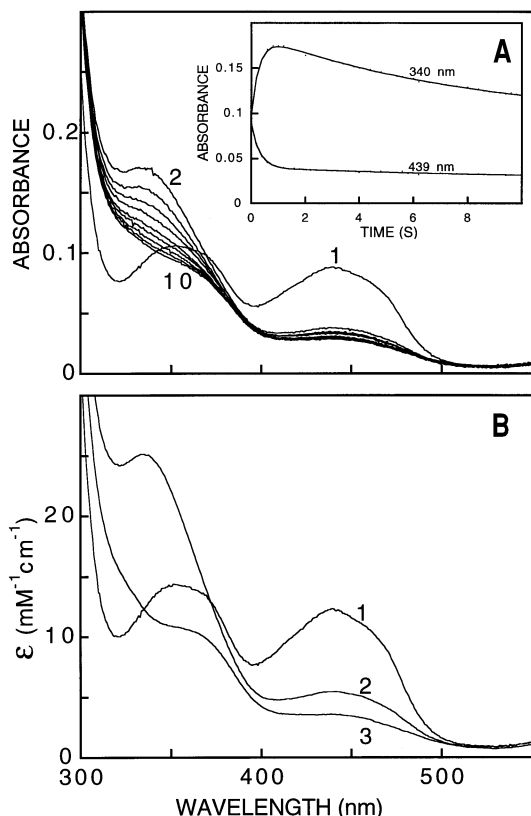


Fig. 3. Spectral changes observed during the reductive half-reaction of vanillyl-alcohol oxidase with 4-ethylphenol. (A) Spectral changes observed upon anaerobic mixing of vanillyl-alcohol oxidase (7.6 μM) with 4-ethylphenol (500 μM) at pH 7.5, 25°C. Original spectral scans are shown from 5.6 ms (1) to 18 s with intervals of 2 s (2–10). The inset shows the original data monitored at 340 nm and 439 nm illustrating the biphasic nature of the reduction reaction. (B) Spectra obtained from deconvolution of spectral changes shown in A. A consecutive irreversible model ($\text{A} \rightarrow \text{B} \rightarrow \text{C}$) was used to fit the data resulting in apparent rates of 3.4 s^{-1} and 0.12 s^{-1} for the two observed reductive phases. Upon reaction of vanillyl-alcohol oxidase with 4-ethylphenol, the original spectrum (1) transforms to (2) and subsequently (3) is formed.

The solvent isotope effect on the rate of enzyme reduction with 4-ethylphenol was also determined. In 50% D_2O , a similar increase in the initial rate of flavin reduction was found as with the steady-state experiments ($k_2 = 2.8 \text{ s}^{-1}$). The origin of this stimulating effect is difficult to explain but confirms that flavin reduction does not involve fission of a hydrogen bond originating from a water molecule. However, the observed stimulation of activity by D_2O may also mask a true solvent isotope effect.

Anaerobic reduction of vanillyl-alcohol oxidase by 4-methylphenol was a slow and apparent monophasic process (Fig. 4). The rate of reduction (k_{fast}) was dependent on the substrate concentration and ranged over 0.11–0.15 s^{-1} (25–250 μM 4-methylphenol). The kinetic data concerning k_{fast} were again analyzed using the procedure of Strickland et al. [17]. The observation that reduction by 4-methylphenol did not lead to fully reduced enzyme is in line with this model which includes a reversible reduction (Eqn 2). A maximal reduction rate of 0.076 s^{-1} (k_2) was determined while the reversible step occurs at a maximal rate of 0.086 s^{-1} (k_{-2}) (Table 2). With 4- $[\alpha\text{-}^2\text{H}_3]$ methylphenol the rate of reduction (k_{fast}) significantly decreased (Fig. 4). Furthermore, the extent of reduction also decreased indicating that the rate of reduction (k_2) decreased to a greater extent than

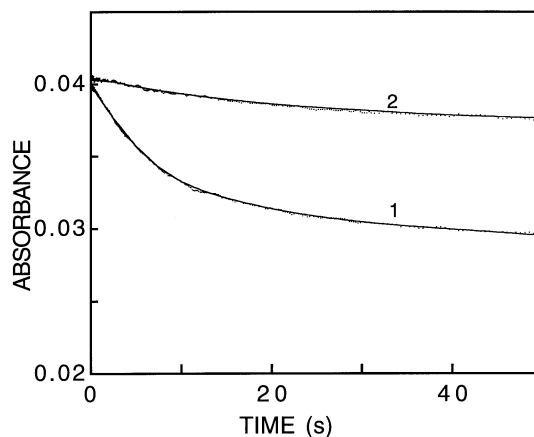


Fig. 4. Absorbance changes observed upon anaerobic reaction of vanillyl-alcohol oxidase with 4-methylphenol. Vanillyl-alcohol oxidase (3.3 μM) was mixed with 75 μM 4-methylphenol (trace 1) or 4- $[\alpha\text{-}^2\text{H}_3]$ methylphenol (trace 2) when monitored at 439 nm, pH 7.5, 25°C. The data could satisfactorily be fitted according to a monophasic process yielding apparent reaction rates of 0.133 s^{-1} (1) and 0.045 s^{-1} (2), respectively.

the rate of the reversible reduction (k_{-2}). Because of the small changes in absorbance and k_{fast} upon changing the substrate concentration, the dissociation constant for the Michaelis complex (k_{-1}/k_1) could not be determined accurately. However, it is expected that this parameter will be in the same range as for the non-deuterated substrate. Again, using the fraction of oxidized enzyme formed as a result of reduction by deuterated 4-methylphenol [corresponding to the ratio of $k_2/(k_{-2} + k_2)$ at 250 μM], k_{-2} could be calculated. Assuming that the dissociation constant for the Michaelis complex is not significantly affected by deuteration, the rate of reduction could be calculated (Table 2). A relatively large isotope effect was seen on the rate of reduction (k_2) while the rate of the reverse reaction was only slightly affected.

Fluorescence studies. The results presented above (cf. Fig. 1) lend strong support to our earlier proposal from X-ray diffraction studies that vanillyl-alcohol oxidase forms an air-stable covalent adduct with 4-methylphenol [9]. The absorption spectrum of the species generated upon reaction of vanillyl-alcohol oxidase with 4-methylphenol at pH 9.4 resembles that of N5 flavin adducts identified in other flavoprotein oxidases [21–23]. Because N5 flavin adducts are fluorescent [21], the nature of the complex between vanillyl-alcohol oxidase and 4-methylphenol was studied in further detail by fluorescence spectroscopy. As can be seen from Fig. 5 (trace 2), the 4-methylphenol-complexed enzyme displayed an intense fluorescence with a broad emission maximum around 475 nm. In contrast, almost no fluorescence was observed with the free oxidized enzyme (Fig. 5, trace 1) or with the enzyme reduced by vanillyl alcohol (not shown). Anaerobic reduction of vanillyl-alcohol oxidase by 4-ethylphenol at pH 7.5 eventually led to the formation of an enzyme species which shows some spectral similarity with the 4-methylphenol adduct (Fig. 3B). This enzyme species was also fluorescent but displayed an emission maximum around 535 nm (Fig. 5, trace 3). A similar fluorescent species accumulated when vanillyl-alcohol oxidase was anaerobically reacted with 4-propylphenol or when the reaction with 4-ethylphenol was performed at pH 9.4. These results suggest that in the second relatively slow phase of the reductive half-reactions of vanillyl-alco-

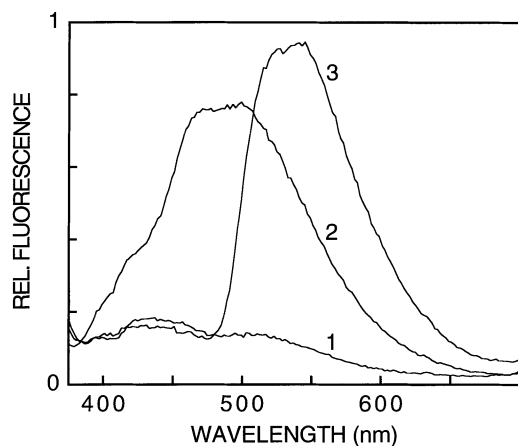


Fig. 5. Fluorescence properties of vanillyl-alcohol oxidase. The fluorescence emission spectra were recorded at 25°C. (1) 10 μ M oxidized vanillyl-alcohol oxidase (pH 7.5), (2) 10 μ M vanillyl-alcohol oxidase, after mixing with 0.5 mM 4-methylphenol (50 mM glycine/NaOH, pH 9.4) and (3) 10 μ M vanillyl-alcohol oxidase, after mixing with 1.0 mM 4-ethylphenol under anaerobic conditions (pH 7.5). The excitation wavelength was 360 nm.

hol oxidase with 4-ethylphenol and 4-propylphenol an N5 flavin adduct is also formed.

DISCUSSION

In this study we have described the kinetic mechanism of vanillyl-alcohol oxidase with short-chain 4-alkylphenols. For the reactions with 4-ethylphenol and 4-propylphenol, it was clearly shown that flavin reduction is rate limiting in overall catalysis. The flavin reduction rates for the non-deuterated and deuterated forms of these substrates are similar to the corresponding k_{cat} values and the enzyme is predominantly in the oxidized state during turnover. During the reductive half-reaction, a transient intermediate is formed which is ascribed to the binary complex between reduced enzyme and the *p*-quinone methide of the alkylphenols. Addition of water to these quinoid species preferentially occurs after flavin reoxidation, followed by product release which completes the catalytic cycle. The kinetic mechanism of vanillyl-alcohol oxidase with 4-ethylphenol and 4-propylphenol is slightly different from that with 4-(methoxymethyl)phenol [6]. With the latter substrate, the *p*-quinone methide intermediate is considerably stabilized in the active site of the reduced enzyme, resulting in a true ternary complex mechanism. With 4-ethylphenol and 4-propylphenol, a more rapid decomposition of the reduced-enzyme-*p*-quinone-methide complex is observed resembling to some extent the sequence of events observed with vanillyl alcohol [6].

The crystal structure of vanillyl-alcohol oxidase has revealed that the active site is located in the interior of the protein and is shielded from solvent [9]. This corresponds to the observed enantioselective hydroxylation of 4-alkylphenols [15], which is indicative for an enzyme-mediated nucleophilic attack of water at the methide carbon. From the crystallographic data we have suggested that charge balancing between the side chain of Arg504 and the anionic reduced flavin might favor the stabilization of the neutral substrate quinone. Upon flavin reoxidation, this charge balancing is lost, facilitating water attack. However, in the enzymatic reactions with 4-ethylphenol and 4-propylphenol, addition of water to the quinone intermediate is less efficient than with 4-(methoxymethyl)phenol and significant amounts of

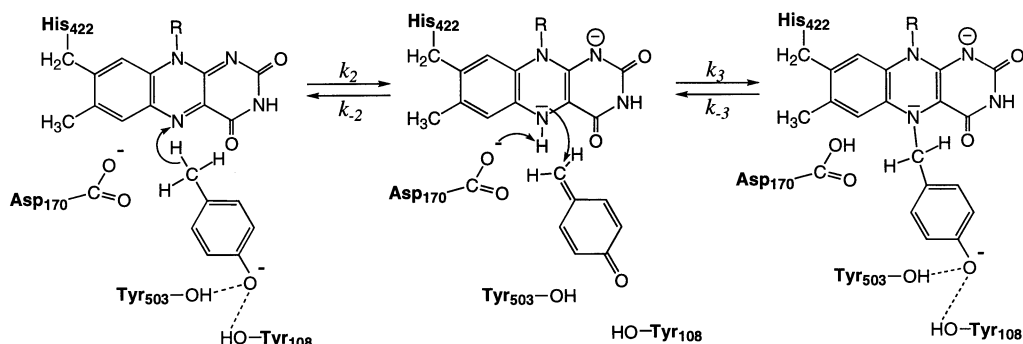
vinylc phenols are formed [15]. It is not clear whether this competing rearrangement of the 4-alkylphenol *p*-quinone methide intermediates is related to the water accessibility of the active site. In this respect it is interesting to note that, in the reaction of vanillyl-alcohol oxidase with 2-methoxy-4-propylphenol, hardly any vinylc product is formed [15].

Reduction of vanillyl-alcohol oxidase by short-chain 4-alkylphenols is not very efficient as the reverse reaction can also proceed at a considerable rate. A similar reversible reduction was recently reported for yeast D-amino acid oxidase [18]. In contrast, with the natural substrate 4-(methoxymethyl)phenol, no significant reverse reduction of vanillyl-alcohol oxidase occurs [6]. For the related enzyme *p*-cresol methylhydroxylase, a reversible reduction of the flavin was also observed upon reaction with 4-ethylphenol, but the rate of reduction was several orders of magnitude higher [24]. With vanillyl-alcohol oxidase and similar to *p*-cresol methylhydroxylase, almost no kinetic deuterium isotope effect on k_{-2} was observed. Apparently, the hydrogen or deuterium abstracted from C α of the substrate during reduction of the flavin is not necessarily involved in the reverse reaction. Possibly, there is an alternative source of hydrogen or the transferred hydrogen may be quickly exchanged. A possible residue involved in this may be Asp170, the side chain of which is located close to the C α atom of the substrate and the N5 atom of the isoalloxazine ring [9].

In the reaction of vanillyl-alcohol oxidase with 4-methylphenol a rather slow reduction of the flavin was observed which was, however, too fast to account for the rate of overall catalysis. Moreover, with this substrate the enzyme is mainly in the reduced state during turnover, particularly at high pH. During the aerobic conversion of 4-methylphenol at pH 9.4, a fluorescent reduced enzyme species was stabilized with spectral features similar to that of 4-methylphenol-soaked vanillyl-alcohol oxidase crystals [9]. From this, and the fact that the spectral properties of the complex between vanillyl-alcohol oxidase and 4-methylphenol compare well with the recently identified FAD adduct in nitroalkane oxidase [23] and flavin adducts observed in lactate oxidase [21, 22], it is proposed that the stabilisation of the reduced form of the enzyme is due to the formation of an air-stable 4-methylphenol-N5-flavin adduct. A possible mechanism which accounts for this adduct formation is presented in Scheme 1. The first step of the proposed mechanism involves the transfer of a hydride from the C α atom of the substrate to the N5 of the isoalloxazine ring. As a result, a binary complex between reduced enzyme and the *p*-quinone methide of 4-methylphenol is formed. In the second step, Asp170 acts as a base by abstracting a proton from flavin N5 thereby activating the cofactor for nucleophilic attack of the *p*-quinone methide intermediate.

Taken together, the results of this study indicate that, during the reaction of 4-methylphenol with vanillyl-alcohol oxidase, an N5 flavin adduct is formed which decomposes only slowly to form a product and hence limits turnover. Crystallographic data have suggested that for more bulky 4-alkylphenols, flavin adduct formation may be prevented by steric constraints [9]. However, the results presented here indicate that with both 4-ethylphenol and 4-propylphenol, an N5 flavin adduct is formed under anaerobic conditions. From this, it is reasonable to assume that $E_{\text{red}} \sim P$ in Eqn (2) represents this adduct. A similar mechanism as shown in Scheme 1 for 4-methylphenol may be operative for flavin adduct formation with 4-ethylphenol and 4-propylphenol. However, with the latter substrates, adduct formation is relatively slow in comparison with flavin reduction. Moreover, during turnover the enzyme is predominantly in the oxidized form. This indicates that, similar to the reaction with 4-(methoxymethyl)phenol [6], dioxygen reacts rapidly with $E_{\text{red}} \sim Q$. From the above

Scheme 1.



considerations it is concluded that in the reaction of vanillyl-alcohol oxidase with 4-ethylphenol and 4-propylphenol, flavin adduct formation is not of catalytic relevance.

Finally, from the results presented in this paper it is clear that vanillyl-alcohol oxidase and *p*-cresol methylhydroxylase differ significantly in their kinetic properties with short-chain 4-alkylphenols. Moreover, both covalent flavoenzymes use different electron acceptors for flavin reoxidation. In vanillyl-alcohol oxidase, the 8 α -(*N*₃-histidyl)-FAD is reoxidized by molecular oxygen while in *p*-cresol methylhydroxylase, reoxidation of the 8 α -*O*-tyrosyl-FAD is accomplished by a fast intramolecular electron transfer to the heme. It has been proposed that this latter process is facilitated by the 8 α -*O*-tyrosyl-FAD phenolic ether bond [11]. Unlike *p*-cresol methylhydroxylase [16] and the related flavocytochrome 4-ethylphenol methylene hydroxylase [25], vanillyl-alcohol oxidase is not involved in the biodegradation of 4-alkylphenols but instead is operative in the metabolism of phenolic methylethers [2]. Therefore, it can be concluded that vanillyl-alcohol oxidase is a true flavoprotein oxidase which fortuitously mediates the (enantioselective) hydroxylation of short-chain 4-alkylphenols via the transient stabilization of their corresponding *p*-quinone methides. With the availability of the vanillyl-alcohol oxidase structure [9] and *vaoA* gene [12], it will be stimulating to unravel the molecular aspects of the substrate specificity and oxygen reactivity of this unusual flavoenzyme in further detail.

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